STIC-ILL

From: Baskar, Padmavathi
Sent: Tuesday, November 05, 2002 11:09 AM
T : STIC-ILL
Subject: 09/833017--DENTAL CARIES

PLEASE PROVIDE ME THE FOLLOWING ASAP. THANK YOU ALL

1. APPL.ENVIRON.MICROBIOL 1998, 64;2247-2255

2. APPL.ENVIRON.MICROBIOL 1999 65; 3710-3713

3. APPL.ENVIRON.MICROBIOL 1996, 62; 2994-2998

4 INFECT.IMMUN, 1983, 41; 722-27

5. INFECT.IMMUN, 1981, 32; 1295-1297

6. J.BACTERIOL, 2000, 182;1374-1382.

Padma Baskar
Art Unit 1645
Patent Examiner/Biotechnology
CM-1, 8E-13, BOX 8E-12
703-308-8886

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1999, p. 3710–3713
0099-2240/99/$04.00+0

Vol. 65, No. 8

High Rates of Conjugation in Bacterial Biofilms as Determined by Quantitative In Situ Analysis

MARTINA HAUSNER AND STEFAN WUERTZ*

Institute of Water Quality Control and Waste Management, Technical University of Munich, D-85748 Garching, Germany

Received 4 January 1999/Accepted 26 April 1999

Quantitative in situ determination of conjugative gene transfer in defined bacterial biofilms using automated confocal laser scanning microscopy followed by three-dimensional analysis of cellular biovolumes revealed conjugation rates 1,000-fold higher than those determined by classical plating techniques. Conjugation events were not affected by nutrient concentration alone but were influenced by time and biofilm structure.

Gene transfer by conjugation is considered to be an important mechanism for the establishment of new genetic traits in diverse environments. Since most bacteria found in nature live in biofilms on surfaces or at interfaces (7), it is likely that conjugation plays an important role in the spread of genetic information (1, 2, 14, 17, 20, 26, 28). Even though the probability of random meetings of cells in suspension is greater than in biofilms, the relative spatial stability of bacteria in biofilms should favor conjugation. Although there may be fewer initial mating events in biofilms, potential mating partners are fixed in a matrix of extracellular polymeric substances and inorganic materials and thus are able to propagate locally. Since frequencies are determined by plating transconjugants on selective media, it remains unclear how high in situ transfer frequencies really are and whether all transconjugants are capable of growth on selective agar plates. Furthermore, the necessity of identifying transconjugant cells by plating procedures involving selective markers such as antibiotic resistance (28), heavy metal resistance (22), or degradative abilities (29) has hampered analysis of the real impact of newly introduced metabolic capacities by, for example, genetically engineered microorganisms on autochthonous microbial populations.

Factors influencing the frequency of gene transfer include cell density (19, 24), growth phase (23), and temperature (11, 16) as well as pH, cations, salinity, dissolved oxygen, and nutrient availability (16, 27). In order to understand the dynamics of in situ gene transfer, we investigated the effects of nutrient concentration; contact time between donors, recipients, and helper cells; and helper cell density. Microscopic observations of the rate of triparental gene transfer involved *Alcaligenes eutrophus* recipient strain AE104 (21), *Escherichia coli* donor strain GM16(pRK415::*gfp*), and *E. coli* helper strain CM404(pRK2013) (12). *A. eutrophus* recipient strain AE104 is a plasmid-free derivative of strain CH34 (22). It has previously been shown to be a good recipient of plasmids and does not appear to have an efficient restriction-modification system. Although the species *A. eutrophus* has recently been reclassified as *Ralstonia eutropha* (31), strain CH34 and its derivatives are different from the type strain of *R. eutropha* (25). Thus, the old classification based on the current recommendation (25) was used.

E. coli donor strain GM16, a derivative of strain DH5α, contained the plasmid pRK415 (15) with a gene for the green fluorescent protein (GFP) from the jellyfish *Aequoria victoria* (4) inserted into the multiple cloning site. This reporter gene construct enabled the determination of gene transfer frequencies independent of the growth of transconjugants. Plasmid pRK415, a derivative of the broad-host-range plasmid RK2, can be transferred from the host when the strain receives the narrow-host-range plasmid pRK2013 (12) which encodes the cognate conjugation system. *E. coli* helper strain CM404 is a derivative of strain HB101 containing pRK2013 (12). Classical conjugation experiments between donor, helper, and recipient strains were performed as previously described (22). Transconjugants and recipients were counted after growth on selective agar media.

To detect gene transfer in biofilms, sterile slides, submerged in 100% Luria-Bertani (LB) medium and inoculated with a single colony of the recipient strain, were incubated in sterile petri dishes at room temperature (RT) on a slowly tilting table. After a 24-h incubation period, the slides were transferred to fresh LB medium (100 or 1%) and approximately 10^8 cells from a culture of the donor strain grown overnight and then washed in phosphate-buffered saline (PBS) (8 g of NaCl liter^{-1}, 0.2 g of KCl liter^{-1}, 1.44 g of Na_2HPO_4 liter^{-1}, 0.2 g of NaH_2PO_4 liter^{-1}) (pH 7.0) and 10^8 or 10^2 cells of the helper strain were added. The slides were again incubated for a contact time period of 2 or 24 h at RT, washed with PBS, and fixed in a solution of PBS plus 4% paraformaldehyde at RT for 1 h. The biofilms on the slides were subjected to ethanol dehydration (50, 80, and 98% ethanol) and hybridized with the rRNA-directed oligonucleotide probe BET42a, labelled with tetramethylrhodamine-5-isothiocyanate (TRITC), specific for the β subgroup of *Proteobacteria* (21). Biofilms were hybridized with 35% formamide in the hybridization solution for 1.5 h at 46°C, washed, and prepared for microscopy as described previously (21).

The hybridized biofilms were investigated with confocal laser scanning microscopy (CLSM) (3) as follows. A series of images in the *z* direction (*z* series) were digitized in selected optical planes with an LSM410 confocal laser scanning microscope (C. Zeiss, Jena, Germany). Image generation was achieved using the 488- and 543-nm-wavelength laser lines, in combination with 515- to 540-nm-wavelength band-pass and 590-nm-wavelength long-pass emission filters for GFP and TRITC fluorescence, respectively. Images were obtained with a 40×/1.3 NA Plan-Neofluar oil immersion lens and a 2.5× digital zoom factor. Transconjugants exhibited both TRITC and GFP fluorescence and were recognized as objects with

* Corresponding author. Mailing address: Institute of Water Quality Control and Waste Management, Technical University of Munich, Am Coulombwall, D-85748 Garching, Germany. Phone: 49 (89) 2891 3708. Fax: 49 (89) 2891 3718. E-mail: stefan.wuertz@bv.tum.de.

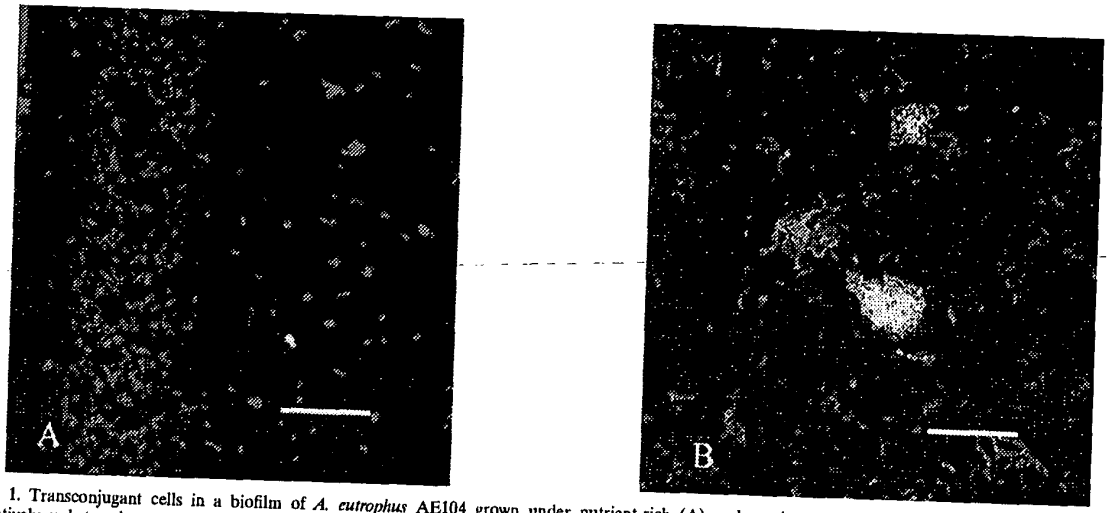

FIG. 1. Transconjugant cells in a biofilm of *A. eutrophus* AE104 grown under nutrient-rich (A) and nutrient-poor (B) conditions. Signals were collected consecutively and stored as grey images. With computer-assisted coloring, recipient AE104 cells were assigned the color red. Donor *E. coli* GM16 cells are depicted in green. Transconjugants emitted both green light (due to GFP) and red light (due to hybridization with the TRITC-labelled rRNA-directed oligonucleotide probe) and are shown in yellow. Bar, 25 μm.

superimposed pixels when GFP and TRITC signals from corresponding images were compared. Typically, transconjugants in biofilms exposed to 100% LB medium occurred as single cells or in pairs (Fig. 1A). In contrast, conjugation in biofilms exposed to 1% LB medium was facilitated by the formation of clusters; transconjugants occurred as subclusters within these aggregates (Fig. 1B). Analysis of microscopic images showed that the average biofilm thickness ranged from 3 to 4 μm, and the highest cell density was detected at a distance of 1.5 to 2 μm from the surface of the glass slide (Fig. 2). No obvious spatial separation of transconjugants, donors, or recipients was observed.

In order to detect rare events in situ and arrive at a quantitative estimate, automated on-line collection of confocal two-dimensional cross-sectional images followed by off-line image processing to determine biovolumes (18) was used. Thus, transfer rates were calculated by determining ratios of transconjugant to recipient cell volume per hour of contact time. Three-way analysis of variance analysis (SigmaStat; Jandel Scientific Software, San Rafael, Calif.) of conjugation rates revealed that helper cell density or nutrient concentration alone had no significant effect on the outcome of the experiments. At first, it may be surprising that the different helper cell densities resulted in similar conjugation frequencies. However, it is likely that the transfer of the pRK415 plasmid is the rate-limiting step. Laboratory results indicate that the transfer frequency of the pRK2013 plasmid from the helper cell to the donor cell is higher than that of pRK415 from the donor cell to the recipient cell (12a). In addition, some helper cells could donate the pRK2013 plasmid directly to the recipient cells, which would then acquire the entry exclusion phenotype, thereby preventing their conjugation with the donor cells. This could also be a plausible explanation for the fact that at higher helper cell densities, rates comparable to those observed with lower helper cell densities were obtained.

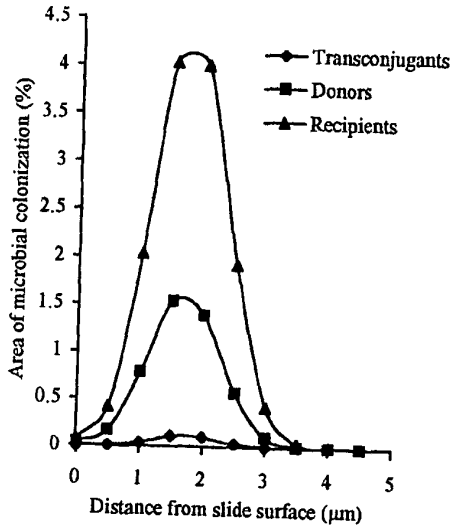

FIG. 2. Average distribution of recipient, donor, and transconjugant cells at different depths in a 7.3×10^4 μm^3 volume of biofilm grown in rich medium for 24 h.

In contrast, time was a significant factor and the effect of nutrient concentration depended on time. The two data sets (10^2 and 10^8 helper cells) were also separately subjected to a two-way analysis of variance followed by the Student-Newman-Keuls pairwise multiple comparison method (Table 1). For both helper cell concentrations, the highest hourly rates were obtained in biofilms with contact time limited to 2 h. Interestingly, for the 10^8 helper cell data set, the highest rate was observed under low nutrient conditions, and similarly, the second highest rate for the 10^2 helper cell set was also obtained in biofilms grown with reduced nutrient concentration. The formation of clusters at low nutrient concentrations may have contributed to the high transfer rates under these conditions. Christensen et al. (5) also observed a positive correlation between transconjugant establishment and the preexisting pool of recipient cells. Additionally, they also found that the frequency of transconjugants first increased and then levelled off during the course of a 6-day experiment as determined in biofilm effluents by selective plating techniques. Surprisingly, in our experiments, the lowest rates were also found under low nutrient conditions but only in those biofilms in which contact

TABLE 1: Triparental gene transfer rates in *A. eutrophus* biofilms as a function of cell contact time, helper cell density, and nutrient concentration

Medium	Contact time (h)	Triparental gene transfer rate in biofilm (no. of transconjugants/recipient cell/h)[a]	
		10^2 helper cells added	10^6 helper cells added
100% LB	2	2.4×10^{-3} A	7.5×10^{-4} E
	24	7.1×10^{-4} AB	9.1×10^{-4} E
1% LB	2	1.2×10^{-3} AB	3.4×10^{-3} D
	24	2.5×10^{-4} B	5.8×10^{-4} E

[a] The total volumes analyzed per treatment were 1.8×10^6 μm^3 for slides with 10^6 helper cells (25 vertical stacks of images with 10 *x-y* optical sections and a volume of 7.3×10^4 μm^3 per stack) and 3.7×10^6 μm^3 for slides with 10^2 helper cells (50 vertical stacks). For each set, means with different letters are significantly different at $P \leq 0.05$ by the Student-Newman-Keuls pairwise multiple comparison method.

time was extended to 24 h. In these biofilms, detachment of recipient cells was observed (results not shown). This may have affected conjugation rates to the extent that fewer *A. eutrophus* AE104 cells were available as recipients. It is also possible that some recipients obtained the pRK2013 helper plasmid directly. As stated above, such transconjugants possess the entry exclusion phenotype and cannot accept the pRK415 plasmid. In this way, the already diminished potential recipient pool could be compromised, resulting in an effective reduction of the frequency of gene transfer.

The observed GFP fluorescence after 2 h contradicts previous results which indicated that the protein should require 4 to 6 h to develop its fluorescence (8, 13). Other workers have reported GFP expression after 13 h as a result of TOL plasmid transfer (6). Using CLSM, we were able to detect even dimly fluorescent signals resulting from incompletely matured GFP protein. Imperfect or incomplete maturation may result in diminished fluorescence and thus raise the threshold number of GFP copies within a cell required for GFP fluorescence to be detectable (30). Control slides containing only recipient cells did not show any fluorescence at the GFP detection settings employed. Maximum rates of 2.4×10^{-3} and 1.2×10^{-3} transconjugants per recipient per hour were determined during a contact time of 2 h for nutrient levels of 100 and 1% LB medium, respectively, when 10^2 helper cells were used (Table 1). These rates are from 1 to >3 orders of magnitude higher than the rates obtained by determining the number of transconjugants based on CFU. The reasons why only a fraction of transconjugant cells were able to form colonies on selective agar plates are unknown. The explanation may be a viable but nonculturable state of transconjugants or a loss of plasmids upon cell division even under selective conditions.

Our results differ significantly from those reported for horizontal plasmid transfer on floating filters from *Pseudomonas putida* to *Vibrio* sp. and *Deleya marina* (9). In that study, gene transfer frequency was expressed as the number of transconjugants per added donor cell, and no difference was found for in situ detection by GFP fluorescence and traditional plating techniques. The reasons for this may be either methodological (in this study, the biovolumes of over 6,000 cells were analyzed with CLSM) or due to more-complex physiological conditions in biofilms (compared to filtered cells on floating filters). For example, in *Pseudomonas aeruginosa* biofilms, the production of chemical signals appears to play a role in developmental patterns (10).

Our technique detects plasmid transfer and expression of GFP but does not allow an evaluation of plasmid stability in the recipient. Clearly, although the rate of transfer can be stimulated by biofilm structure, the establishment of new genetic traits depends on plasmid stability in the new host. Obviously, selective pressures in the form of metabolic requirements for degradation of toxic compounds can have an additional effect, which may increase the rate of second-generation gene transfer. It is puzzling in this respect that a study which dealt with in situ gene transfer using GFP did not report any effect of the toxic metal Hg on the dissemination of an indigenous plasmid carrying genes conferring resistance to Hg (9).

Conjugation is an energy-demanding process; however, if the conjugation machinery is constitutively expressed at starvation, no extra energy is needed for starved cells to donate their DNA. Nutrient limitation does not seriously impede conjugation in natural systems, and even starved cells are capable of transferring plasmids (9). Our results have clearly demonstrated that conjugation rates are not diminished by a reduction in nutrient concentration. In the light of these observations, it would be of interest to investigate the effect of biofilm structure on conjugative gene transfer. The implications of the results of this study are that initial gene transfer in biofilms occurs far more frequently than previously thought. The knowledge that attached cells of the same species differ in their ability to maintain incoming plasmids hints at specific physiological conditions in biofilms which lead to individual cells experiencing different environmental pressures. We have provided the first quantitative glimpse at conjugative events in biofilms at the single-cell level which in the future should allow one to influence the incidence of genetic exchange by affecting biofilm structure itself.

We thank G. Weber for discussions concerning statistical analyses. We are grateful to Pierre Wattiau for critically reading the manuscript. Strains AE104 and CM404 were donated by Dirk Springael, and pRK415 was obtained from Annick Wilmotte.

This work was supported in part by the Research Center for Fundamental Studies of Aerobic Biological Wastewater Treatment, Munich, Germany (SFB411).

REFERENCES

1. **Ashelford, K. E., J. C. Fry, and M. A. Learner.** 1998. Plasmid transfer between strains of *Pseudomonas putida*, and their survival, within a pilot scale percolating-filter sewage treatment system. FEMS Microbiol. Ecol. 18:15–26.
2. **Bale, M. J., J. C. Fry, and M. J. Day.** 1987. Plasmid transfer between strains of *Pseudomonas aeruginosa* on membrane filters attached to river stones. J. Gen. Microbiol. 133:3099–3107.
3. **Caldwell, D. E., D. R. Korber, and J. R. Lawrence.** 1992. Confocal laser scanning microscopy and digital image analysis in microbial ecology. Adv. Microb. Ecol. 12:1–67.
4. **Chalfie, M., Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher.** 1994. Green fluorescent protein as a marker for gene expression. Science 263:802–804.
5. **Christensen, B. B., C. Sternberg, J. B. Andersen, L. Eberl, S. Moller, M. Givskov, and S. Molin.** 1998. Establishment of new genetic traits in a microbial biofilm community. Appl. Environ. Microbiol. 64:2247–2255.
6. **Christensen, B. B., C. Sternberg, and S. Molin.** 1996. Bacterial plasmid conjugation on semi-solid surfaces monitored with the green fluorescent protein (GFP) from *Aequoria victoria* as a marker. Gene 173:59–65.
7. **Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott.** 1995. Microbial biofilms. Annu. Rev. Microbiol. 49:711–745.
8. **Cubitt, A. B., R. Hiem, S. R. Adams, A. E. Boyd, L. A. Gross, and R. Y. Tsien.** 1995. Understanding, improving and using green fluorescent proteins. Trends Biochem. Sci. 20:448–455.
9. **Dahlberg, C., M. Bergström, and M. Hermansson.** 1998. In situ detection of high levels of horizontal plasmid transfer in marine bacterial communities. Appl. Environ. Microbiol. 64:2670–2675.
10. **Davies, D. G., M. R. Parsek, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of biofilms. Science 280:295–298.

11. **Day, M. G., J. C. Fry, and J. Diaper.** 1992. Effect of host cell physiology on plasmid transfer in river epilithon, p. 35–50. *In* M. J. Gauthier (ed.), Gene. transfers and environment. Springer-Verlag, Berlin, Germany.
12. **Figurski, D. H., and D. H. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on plasmid function provided in trans. Proc. Natl. Acad. Sci. USA 76:1648–1652.
12a. **Hausner, M., and S. Wuertz.** Unpublished data.
13. **Heim, R., A. B. Cubitt, and R. Y. Tsien.** 1995. Improved green fluorescence. Nature 373:663–664.
14. **Hoffmann, A., T. Thimm, M. Dröge, E. R. B. Moore, J. C. Munch, and C. C. Tebbe.** 1998. Intergeneric transfer of conjugative and mobilizable plasmids harbored by *Escherichia coli* in the gut of the soil microarthropod *Folsomia candida* (Collembola). Appl. Environ. Microbiol. 64:2652–2659.
15. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene 70:191–197.
16. **Khalil, T. A., and M. A. Gealt.** 1987. Temperature, pH, and cations affect the ability of *Escherichia coli* to mobilize plasmids in L broth and synthetic waste water. Can. J. Microbiol. 33:733–737.
17. **Kroer, N., T. Barkay, S. Sorensen, and D. Weber.** 1998. Effect of root exudates and bacterial metabolic activity on conjugal gene transfer in the rhizophere of a marsh plant. FEMS Microbiol. Ecol. 25:375–384.
18. **Kuehn, M., M. Hausner, H.-J. Bungartz, M. Wagner, P. A. Wilderer, and S. Wuertz.** 1998. Automated confocal laser scanning microscopy and semiautomated image processing for analysis of biofilms. Appl. Environ. Microbiol. 64:4115–4127.
19. **Levin, B. R., F. M. Stewart, and V. A. Rice.** 1979. The kinetics of conjugative plasmid transmission: fit of a simple mass action model. Plasmid 2:247–260.
20. **Lilley, A. K., and M. J. Bailey.** 1997. The acquisition of indigenous plasmids by a genetically marked pseudomonad population colonizing the sugar beet phytosphere is related to local environment conditions. Appl. Environ. Microbiol. 63:1577–1583.
21. **Manz, W., U. Szewzyk, P. Ericksson, R. Amann, K. H. Schleifer, and T. A. Stenström.** 1993. In situ identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S and 23S rRNA-directed fluorescent oligonucleotide probes. Appl. Environ. Microbiol. 59:2293–2298.
22. **Mergeay, M., D. Nies, H. G. Schlegel, J. Gerits, P. Charles, and F. Van Gijsegem.** 1985. *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J. Bacteriol. 162:328–334.
23. **Muela, A., M. Pocino, I. Arana, J. Justo, J. Irreberri, and I. Barcina.** 1994. Effect of growth phase and parental cell survival in river water on plasmid transfer between *Escherichia coli* strains. Appl. Environ. Microbiol. 60:4273–4278.
24. **Normander, B., B. B. Christensen, S. Molin, and N. Kroer.** 1998. Effect of bacterial distribution and activity on conjugal gene transfer on the phylloplane of the bush bean (*Phaseolus vulgaris*). Appl. Environ. Microbiol. 64:1902–1909.
25. **Peitzsch, N., G. Eberz, and D. H. Nies.** 1998. *Alcaligenes eutrophus* as a bacterial chromate sensor. Appl. Environ. Microbiol. 64:453–458.
26. **Ravatn, R., A. J. B. Zehnder, and J. R. van der Meer.** 1998. Low-frequency horizontal transfer of an element containing the chlorocatechol degradation genes from *Pseudomonas* sp. strain B13 to *Pseudomonas putida* F1 and to indigenous bacteria in laboratory-scale activated-sludge microcosms. Appl. Environ. Microbiol. 64:2126–2132.
27. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. 51:365–379.
28. **Sandaa, R., and O. Enger.** 1994. Transfer in marine sediments of the naturally occurring plasmid pRAS1 encoding multiple antibiotic resistance. Appl. Environ. Microbiol. 60:3430–3437.
29. **Springael, D., L. Diels, L. Hooyberghs, S. Kreps, and M. Mergeay.** 1993. Construction and characterization of heavy metal-resistant haloaromatic-degrading *Alcaligenes eutrophus* strains. Appl. Environ. Microbiol. 59:334–339.
30. **Tsien, R.** 1998. The green fluorescent protein. Annu. Rev. Biochem. 67:509–544.
31. **Yabuuchi, E., Y. Kosako, I. Yano, H. Hotta, and Y. Nishiuchi.** 1995. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith, 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. Microbiol. Immunol. 39:897–904.